ARGININE REPRESSOR DEFICIENT STRAIN OF CORYNEFORM BACTERIUM AND METHOD FOR PRODUCING L-ARGININE

Field of the Invention

5   The present invention relates to a coryneform bacterium having an ability to produce L-arginine and a method for producing L-arginine using the bacterium. L-arginine is an industrially useful amino acid as an ingredient of liver function promoting agents, amino
10   acid infusions, comprehensive amino acid pharmaceuticals and so forth.

Description of the Related Art

Conventional L-arginine production by
15   fermentation has been performed by utilizing wild-type strains of coryneform bacteria; coryneform bacteria resistant to certain agents including sulfa drugs, 2-thiazolealanine, á-amino-â-hydroxyvaleric acid and the like; coryneform bacteria exhibiting auxotrophy for L-
20   histidine, L-proline, L-threonine, L-isoleucine, L-methionine, or L-tryptophan in addition to the resistance to 2-thiazolealanine (Japanese Patent Laid-open No. 54-44096); coryneform bacteria resistant to ketomalonic acid, fluoromalonic acid, or
25   monofluoroacetic acid (Japanese Patent Laid-open No. 57-18989); coryneform bacteria resistant to argininol (Japanese Patent Laid-open No. 62-24075); coryneform

bacteria resistant to X-guanidine (X represents a derivative of fatty acid or aliphatic chain, Japanese Patent Laid-open No. 2-186995) or the like.

On the other hand, there have also been disclosed methods for producin g L-arginine utilizing recombinant DNA techniques. That is, there has been disclosed a method for producing L-arginine by utilizing a microorganism belonging to the genus *Corynebacterium* or *Brevibacterium* which is made to harbor a recombinant DNA comprising a vector DNA and a DNA fragment containing genes for acetylornithine deacetylase, N-acetylglutamic acid-ā-semialdehyde dehydrogenase, N-acetyl glutamokinase, and argininosuccinase derived from a microorganism belonging to the genus *Escherichia* (Japanese Patent Publication No. 5-23750).

Further, as for coryneform bacteria, it has been elucidated that synthesis of some enzymes of the L-arginine biosynthetic system is repressed by L-arginine. Furthermore, it was reported that, while some of enzymes of L-arginine biosynthetic system were repressed by L-arginine, the repression of these enzymes by L-arginine was canceled in mutant strains of coryneform bacteria showing improved L-arginine accumulation amounts (*Agric. Biol. Chem.*, *43*(1), 105, 1979).

Meanwhile, as for *Escherichia coli*, a repressor of L-arginine biosynthetic system and a gene coding for the repressor were identified (*Proc. Natl. Acad. Sci. U.S.A.*

(1987), *84*(19), 6697-701), and binding interactions of the repressor protein and various genes of L-arginine biosynthetic system were also investigated (*Proc. Natl. Acad. Sci. U.S.A.* (1987), *84*(19), 6697-701, *J. Mol. Biol.* (1992), *226*, 367-386).

However, any repressor proteins of the L-arginine biosynthetic system have not been identified in coryneform bacteria. While a nucleotide sequence of the repressor protein gene (*argR*) and an amino acid sequence assumed to be encoded thereby are registered in a gene database, GenBank (AF049897), the gene is considered to be designated *argR* because of the homology between the aforementioned amino acid sequence and known arginine repressors.

Summary of the Invention

As described above, although a repressor protein of the L-arginine biosynthetic system of coryneform bacteria and a gene thereof are expected, the repressor protein itself has not been identified and its functions and so forth are not elucidated at all. Therefore, an object of the present invention is to identify the repressor of the L-arginine biosynthesis in coryneform bacteria, and improve L-arginine productivity of coryneform bacteria.

The inventors of the present invention isolated a

homologue of the gene registered as *argR* in the gene
database (GenBank accession AF049897) from a coryneform
bacterium, and found that, if this gene was amplified in
coryneform bacteria, L-arginine producing ability was

5 decreased, and on the other hand, if the gene was
disrupted, the L-arginine producing ability was improved,
to confirm that the L-arginine biosynthesis is repressed
by a repressor in coryneform bacteria like *Escherichia
coli* and the aforementioned gene registered as *argR*

10 codes for the repressor. Thus, the present invention
was accomplished.

That is, the present invention provides the
followings.

(1) A coryneform bacterium in which an arginine

15 repressor does not function in a normal manner, and
which has L-arginine producing ability.

(2) The coryneform bacterium according to (1), wherein
the arginine repressor does not function in a normal
manner due to disruption of a gene coding for the

20 arginine repressor on a chromosome of the bacterium.

(3) The coryneform bacterium according to (2), wherein
the arginine repressor has the amino acid sequence shown
in SEQ ID NO: 18 or an amino acid sequence showing
homology to the amino acid sequence.

25 (4) A method for producing L-arginine, which comprises
culturing a coryneform bacterium according to any one of
(1) to (3) in a medium to produce and accumulate L-

arginine in the medium, and collecting the L-arginine from the medium.

In the present invention, the "arginine repressor" refers to a protein that has an effect of repressing the L-arginine biosynthesis, and if expression amount of the gene that codes for the protein increases in coryneform bacteria, L-arginine producing ability will be reduced, and if the expression amount decreases or the protein disappears, the L-arginine producing ability will be improved. Hereafter, the gene coding for the arginine repressor is also called *argR* gene. Further, the "L-arginine producing ability" used in the present invention refers to an ability of the microorganism of the present invention to accumulate L-arginine in a medium, when it is cultured in the medium.

According to the present invention, L-arginine producing ability of coryneform bacteria having the L-arginine producing ability can be improved.

Brief Explanation of the Drawings

Fig. 1 shows the construction process of plasmid pK1.

Fig. 2 shows the construction process of plasmid pSFK6.

Fig. 3 shows the construction process of plasmid pSFKT2.

Hereafter, the present invention will be explained in detail.

The microorganism of the present invention is a coryneform bacterium having L-arginine producing ability, in which arginine repressor does not function in a normal manner. The coryneform bacterium of the present invention may be a microorganism having the L-arginine producing ability because an arginine repressor does not function in a normal manner in it, or a microorganism bred so that the arginine repressor should not function in a normal manner in it. Alternatively, it may be a microorganism that is bred so that the arginine repressor should not function in a normal manner in it and then imparted with the L-arginine producing ability.

The coryneform bacteria include bacteria having been hitherto classified into the genus *Brevibacterium* but united into the genus *Corynebacterium* at present (*Int. J. Syst. Bacteriol.*, *41*, 255 (1981)), and include bacteria belonging to the genus *Brevibacterium* closely relative to the genus *Corynebacterium*. Examples of such coryneform bacteria include the followings.

Corynebacterium acetoacidophilum

Corynebacterium acetoglutamicum

Corynebacterium alkanolyticum

Corynebacterium callunae

Corynebacterium glutamicum

Corynebacterium lilium (*Corynebacterium glutamicum*)

Corynebacterium melassecola

Corynebacterium thermoaminogenes

5   *Corynebacterium herculis*

Brevibacterium divaricatum (*Corynebacterium glutamicum*)

Brevibacterium flavum (*Corynebacterium glutamicum*)

Brevibacterium immariophilum

10   *Brevibacterium lactofermentum* (*Corynebacterium glutamicum*)

Brevibacterium roseum

Brevibacterium saccharolyticum

Brevibacterium thiogenitalis

15   *Brevibacterium album*

Brevibacterium cerinum

Microbacterium ammoniaphilum

While the coryneform bacteria that have the L-arginine-producing ability are not particularly limited

20   so long as they have the L-arginine-producing ability, they include, for example, wild-type strains of coryneform bacteria; coryneform bacteria resistant to certain agents including sulfa drugs, 2-thiazolealanine, α-amino-β-hydroxyvaleric acid and the like; coryneform

25   bacteria exhibiting auxotrophy for L-histidine, L-proline, L-threonine, L-isoleucine, L-methionine, or L-tryptophan in addition to the resistance to 2-

thiazolealanine (Japanese Patent Laid-open No. 54-44096); coryneform bacteria resistant to ketomalonic acid, fluoromalonic acid, or monofluoroacetic acid (Japanese Patent Laid-open No. 57-18989); coryneform

5 bacteria resistant to argininol (Japanese Patent Laid-open No. 62-24075); coryneform bacteria resistant to X-guanidine (X represents a derivative of fatty acid or aliphatic chain, Japanese Patent Laid-open No. 2-186995) and the like.

10 Specifically, the following strains can be exemplified.

Brevibacterium flavum AJ11169 (BP-6892)

Corynebacterium glutamicum AJ12092 (FERM BP-6906)

Brevibacterium flavum AJ11336 (FERM BP-6893)

15 *Brevibacterium flavum* AJ11345 (FERM BP-6894)

Corynebacterium glutamicum AJ12430 (FERM BP-2228)

The AJ11169 strain and the AJ12092 strain are the 2-thiazolealanine resistant strains mentioned in Japanese Patent Laid-open No. 54-44096, the AJ11336

20 strain is the strain having argininol resistance and sulfadiazine resistance mentioned in Japanese Patent Publication No. 62-24075, the AJ11345 strain is the strain having argininol resistance, 2-thiazolealanine resistance, sulfaguanidine resistance, and exhibiting

25 histidine auxotrophy mentioned in Japanese Patent Publication No. 62-24075, and the AJ12430 strain is the strain having octylguanidine resistance and 2-

thiazolealanine resistance mentioned in Japanese Patent Laid-open No. 2-186995.

AJ11169 was deposited on August 3, 1977 in National Institute of Bioscience and Human Technology, Agency of Industrial Science and Technology, Ministry of International Trade and Industry (currently National Institute of Bioscience and Human Technology, National Institute of Advanced Industrial Science and Technology, Ministry of Economy, Trade and Industry)(zip code: 305-8566, 1-3 Higashi 1-Chome, Tsukuba-shi, Ibaraki-ken, Japan), as deposition number of FERM P-4161, and transferred from the original deposit to international deposit based on Budapest Treaty on September 27, 1999, and has been deposited as deposition number of FERM BP-6892.

AJ12092 was deposited on September 29, 1983 in National Institute of Bioscience and Human Technology, Agency of Industrial Science and Technology, Ministry of International Trade and Industry, as deposition number of FERM P-7273, and transferred from the original deposit to international deposit based on Budapest Treaty on October 1, 1999, and has been deposited as deposition number of FERM BP-6906.

AJ11336 was deposited on April 25, 1979 in National Institute of Bioscience and Human Technology, Agency of Industrial Science and Technology, Ministry of International Trade and Industry, as deposition number

of FERM P-4939, and transferred from the original

deposit to international deposit based on Budapest

Treaty on September 27, 1999, and has been deposited as

deposition number of FERM BP-6893.

5   AJ11345 was deposited on April 25, 1979 in

National Institute of Bioscience and Human Technology,

Agency of Industrial Science and Technology, Ministry of

International Trade and Industry, as deposition number

of FERM P-4948, and transferred from the original

10   deposit to international deposit based on Budapest

Treaty on September 27, 1999, and has been deposited as

deposition number of FERM BP-6894.

        AJ12430 was deposited on December 26, 1988 in

National Institute of Bioscience and Human Technology,

15   Agency of Industrial Science and Technology of Ministry,

International Trade and Industry based on Budapest

Treaty, as deposition number of FERM BP-2228.

        The coryneform bacterium whose arginine repressor

does not function in a normal manner can be obtained by

20   modifying its *argR* gene so that the activity of the

arginine repressor should be reduced or eliminated, or

the transcription of the *argR* gene should be reduced or

eliminated. Such a coryneform bacterium can be obtained

by, for example, replacing the chromosomal *argR* gene

25   with an *argR* gene that does not function in a normal

manner (occasionally referred to as "disrupted *argR*

gene" hereinafter) through, for example, homologous

recombination based on genetic recombination techniques (Experiments in Molecular Genetics, Cold Spring Harbor Laboratory Press (1972); Matsuyama, S. and Mizushima, S., *J. Bacteriol.*, *162*, 1196 (1985)).

5   In the homologous recombination, when a plasmid carrying a sequence exhibiting homology with a chromosomal sequence or the like is introduced into a corresponding bacterial cell, recombination occurs at a site of the homologous sequence at a certain frequency,

10   and thus the introduced plasmid as a whole is integrated into the chromosome. Then, by causing recombination again at the site of the homologous sequence in the chromosome, the plasmid may be removed from the chromosome. However, depending on the position at which

15   the recombination is caused, the disrupted gene may remain on the chromosome, while the original normal gene may be removed from the chromosome together with the plasmid. By selecting such a bacterial strain, a bacterial strain in which the normal *argR* gene is

20   replaced with a disrupted *argR* gene can be obtained.

Such a gene disruption technique based on the homologous recombination has already been established, and a method utilizing a linear DNA, method utilizing temperature sensitive plasmid or the like can be used

25   therefor. The *argR* gene can also be disrupted by using a plasmid that contains the *argR* gene inserted with a marker gene such as drug resistance gene, and cannot

replicate in a target cell of the coryneform bacterium. That is, in a transformant that has been transformed with such a plasmid and hence acquired drug resistance, the marker gene is integrated in a chromosome DNA. It

5   is likely that this marker gene has been integrated by homologous recombination of the *argR* gene present at the both sides of the marker with the *argR* on the chromosome, and therefore a gene-disrupted strain can efficiently be selected.

10   Specifically, a disrupted *argR* gene used for the gene disruption can be obtained by deletion of a certain region of *argR* gene by means of digestion with restriction exzyme(s) and religation; by insertion of another DNA fragment (marker gene etc.) into the *argR*

15   gene, by introducing substitution, deletion, insertion, addition or inversion of one or more nucleotides in a nucleotide sequence of coding region of *argR* gene, its promoter region or the like by means of site-specific mutagenesis (Kramer, W. and Frits, H. J., Methods in

20   Enzymology, *154*, 350 (1987)) or treatment with a chemical reagent such as sodium hyposulfite and hydroxylamine (Shortle, D. and Nathans, D., *Proc. Natl. Acad. Sci. U.S.A.*, *75*, 270(1978)) or the like, so that the activity of the encoded repressor should be reduced

25   or eliminated, or transcription of the *argR* gene should be reduced or eliminated. Among these embodiments, a method utilizing deletion of a certain region of the

argR gene by digestion with a restriction enzyme and religation, or insertion of another DNA fragment into the *argR* gene is preferred in view of reliability and stability.

5   A plasmid for the *argR* gene disruption can be produced by performing PCR (polymerase chain reaction) using a plasmid containing the *argR* gene and its flanking regions as a template and primers corresponding the terminal portions or franking regions of the *argR*

10   gene to amplify a portion except for an internal portion or the whole portion of the *argR* gene, and cyclizing the obtained amplified product. In the examples mentioned hereinafter, the *argR* gene was disrupted by this method.

      The *argR* gene can be obtained from a chromosomal

15   DNA of a coryneform bacterium by PCR using oligonucleotides prepared based on known nucleotide sequences of the *argR* gene as primers. The *argR* gene can also be obtained from a chromosome DNA library of a microorganism which has a purine operon by a

20   hybridization technique using an oligonucleotide prepared based on a known nucleotide sequence of the *argR* gene as a probe. For the purpose of the present invention, because the *argR* gene is used for preparing a disrupted *argR* gene, it is not necessarily required to

25   contain the full length, and it may contain a length required to cause gene disruption.

      The origin of the *argR* gene is not particularly

limited, so long as it has such a degree of homology
that it should cause homologous recombination with the
argR gene of coryneform bacteria. Specifically, the
argR gene of the *Brevibacterium flavum*, which has the

5   nucleotide sequence shown in SEQ ID NO: 17, and the *argR*
gene of *Corynebacterium glutamicum* (GenBank accession
AF049897) can be mentioned as the *argR* genes of
coryneform bacteria. These *argR* genes are highly
homologous, and it is considered that even an *argR* gene

10 of coryneform bacterium of a genus or species different
from that of a coryneform bacterium of which *argR* gene
is to be disrupted may also be used for the gene
disruption.

In the present invention, the amino acid sequence

15 shown in SEQ ID NO: 18 or an amino acid sequence
exhibiting homology to the amino acid sequence means an
amino acid sequence that is encoded by an *argR* gene
having such a degree of homology that it should cause
homologous recombination with the *argR* gene coding to

20 the amino acid sequence shown in SEQ ID NO: 18 (for
example, an *argR* gene having the nucleotide sequence
shown in SEQ ID NO: 17).

As the primers used for PCR, any primers that
allow amplification of the *argR* gene can be used.

25 Specific examples thereof include the oligonucleotides
having the nucleotide sequences shown in SEQ ID NOS: 19
and 20.

Further, examples of marker gene include drug resistance genes such as a kanamycin resistance gene. A kanamycin resistance gene can be obtained by PCR amplification from a known plasmid containing a

5 kanamycin resistance gene of *Streptococcus faecalis*, for example, pDG783 (Anne-Marie Guerout-Fleury *et al.*, *Gene*, *167*, 335-337 (1995)).

When a drug resistance gene is used as the marker gene, an *argR* gene-disrupted strain can be obtained by

10 inserting the drug resistance gene into a suitable site of the *argR* gene carried by a plasmid, transforming a microorganism with the plasmid, and selecting a drug resistant transformant. Disruption of *argR* gene on a chromosome can be confirmed by analyzing the *argR* gene

15 or the marker gene on the chromosome by Southern blotting, PCR, or the like. Integration of the kanamycin resistance gene into a chromosomal DNA can be confirmed by PCR using primers that allow amplification of the kanamycin resistance gene (e.g., oligonucleotides

20 having nucleotide sequences shown in SEQ ID NOS: 1 and 2).

L-arginine can be efficiently produced by culturing a coryneform bacterium having L-arginine producing ability obtained as described above, in which

25 an arginine repressor does not function in a normal manner, in a medium to produce and accumulate L-arginine in the medium, and collecting the L-arginine from the

medium.

The medium to be used may be selected from well-known media conventionally used for fermentative production of amino acids utilizing microorganisms. That is, it may be a usual medium that contains a carbon source, nitrogen source, inorganic ions, and other organic ingredients as required.

As the carbon source, there can be used saccharides such as glucose, sucrose, lactose, galactose, fructose or starch hydrolysate, alcohols such as glycerol or sorbitol, or organic acids such as fumaric acid, citric acid or succinic acid.

As the nitrogen source, there can be used inorganic ammonium salts such as ammonium sulfate, ammonium chloride or ammonium phosphate, organic nitrogen such as soybean protein hydrolysate, ammonia gas, aqueous ammonia and so forth.

It is desirable to add required substances such as vitamin B_1 and L-homoserine, yeast extract and so forth to the medium in appropriate amounts as organic trace nutrients. Other than the above, potassium phosphate, magnesium sulfate, iron ion, manganese ion and so forth are added in small amounts as required.

The culture is preferably carried out under an aerobic condition for 1-7 days. The culture temperature is preferably controlled to be 24°C to 37°C, and pH is preferably controlled to be 5-9 during the culture.

Inorganic or organic, acidic, alkaline substances, or ammonia gas and so forth can be used for pH adjustment. L-arginine can be collected from the fermentation broth usually by a combination of well-known techniques such as ion exchange resin techniques and other techniques.

Best Mode for Carrying out the Invention

Hereafter, the present invention will be explained more specifically with reference to the following examples.

Example 1: Constructions of shuttle vector for *Escherichia coli* and coryneform bacteria and temperature sensitive vector

First, a vector for introducing an *argR* gene into coryneform bacteria and a temperature sensitive vector for producing an *argR* deficient strain of coryneform bacterium were constructed.

<1> Construction of vector having drug resistance gene of *Streptococcus faecalis*

The kanamycin resistance gene of *Streptococcus faecalis* was amplified by PCR from a known plasmid containing the gene. The nucleotide sequence of the kanamycin resistance gene of *Streptococcus faecalis* has

already been elucidated (Trieu-Cuot, P. and Courvalin, P.: *Gene*, *23* (3), 331-341 (1983)). Based on this sequence, the primers shown in SEQ ID NOS: 1 and 2 were synthesized, and PCR was performed by using pDG783

5 (Anne-Marie Guerout-Fleury, *et al*., *Gene*, *167*, 335-337 (1995)) as a template to amplify a DNA fragment containing the kanamycin resistance gene and its promoter.

The aforementioned DNA fragment was purified by

10 using SUPREC02 produced by Takara Shuzo Co., Ltd., and then completely digested with restriction enzymes *Hind*III and *Hinc*II to be blunt-ended. The blunt-ending was performed by using Blunting Kit produced by Takara Shuzo Co., Ltd. This DNA fragment was mixed with a DNA

15 fragment obtained by purification and blunt-ending of an amplification product of PCR performed by using the primers shown in SEQ ID NOS: 3 and 4 and pHSG399 (see S. Takeshita, *et al*.: *Gene*, *61*, 63-74 (1987)) as a template, and ligated both fragments. The ligation was performed

20 by using DNA Ligation Kit Ver. 2 produced by Takara Shuzo Co., Ltd. Competent cells of *Escherichia coli* JM109 (produced by Takara Shuzo Co., Ltd.) were transformed with the ligated DNA, plated on L medium (10 g/L of Bacto trypton, 5 g/L of Bacto yeast extract, 5

25 g/L of NaCl, 15 g/L of agar, pH 7.2) containing 10 μg/ml of IPTG (isopropyl-â-D-thiogalactopyranoside), 40 μg/ml of X-Gal (5-bromo-4-chloro-3-indolyl-â-D-galactoside)

and 25 μg/ml of kanamycin, and cultured overnight. The emerged blue colonies were picked up, and separated into single colonies to obtain transformant strains.

Plasmids were prepared from the transformant
5   strains by the alkali method (Seibutsu Kogaku Jikkensyo (Text for Bioengineering Experiments), Edited by the Society for Bioscience and Bioengineering, Japan, p.105, Baifukan, 1992), and restriction maps were prepared. One having a restriction map equivalent to that of Fig.
10   1 was designated as pK1. This plasmid is stably harbored in *Escherichia coli*, and imparts kanamycin resistance to a host. Moreover, since it contains the lacZ' gene, it is suitably used as a cloning vector.

15   <2> Construction of shuttle vector pSFK6

As a material for obtaining a temperature sensitive replication control region, a plasmid vector autonomously replicable in both of *Escherichia coli* cells and coryneform bacteria cells was prepared. The
20   plasmid pAM330 extracted from Brevibacterium lactofermentum ATCC13869 [see Japanese Patent Publication Laid-open (Kokai) No. 58-67699] was completely digested with a restriction enzyme *Hind*III, and blunt-ended. This fragment was ligated to a
25   fragment obtained by completely digesting the aforementioned pK1 with a restriction enzyme *Bsa*AI. Brevibacterium lactofermentum ATCC13869 was transformed

with the ligated DNA. The transformation was performed by the electric pulse method [see Japanese Patent Publication Laid-open (Kokai) No. 2-207791].

Transformants were selected on an M-CM2B plate (10 g/L

5   of polypeptone, 10 g/L of yeast extract, 5 g/L of NaCl, 10 μg/L of biotin, 15 g/L of agar, pH 7.2) containing 25 μg/ml of kanamycin. After cultivation for 2 days, colonies were picked up, and separated into single colonies to obtain the transformants. Plasmid DNAs were

10   prepared from the transformants, and restriction maps were prepared. One having the same restriction map as that of Fig. 2 was designated as pSFK6. This plasmid is autonomously replicable in both of *Escherichia coli* and coryneform bacteria, and imparts kanamycin resistance to

15   a host.

<3> Construction of a plasmid having temperature sensitive replication control region

pSFK6 was treated with hydroxylamine *in vitro*.

20   The hydroxylamine treatment was performed according to a known method [see, for example, G.O. Humpherys, *et al.*, *Molec. Gen. Genet.*, *145*, 101-108 (1976)]. DNA undergone the treatment was collected and used for transformation of Brevibacterium lactofermentum ATCC13869 strain. The

25   transformants were selected at a low temperature (25°C) on a CM2B plate containing 25 μg/ml of kanamycin. The appeared transformants were replicated to a similar

selection plate, and cultured at an elevated temperature (34°C). One strain that could not grow on the selection plate containing kanamycin at the elevated temperature was obtained. From this strain, a plasmid was recovered

5   and designated as p48K.

<4> Determination of nucleotide sequence of temperature sensitive replication control region

Nucleotide sequences of replication control region

10   segments in the plasmid pSFK6 having a wild-type replication control region and the plasmid p48K having a temperature sensitive replication control region were determined. The nucleotide sequences were determined on a fully automatic sequencer, ABI310 (ABI), by using DNA

15   Sequencing Kit from ABI. As a result, it was found that there were 6 nucleotide substitutions between the wild-type replication control region and the temperature sensitive replication control region. The nucleotide sequence of the temperature sensitive replication

20   control region segment contained in pSFK6 (derived from full sequence of pAM330), which functions in coryneform bacteria, is shown in SEQ ID NO: 5, and the nucleotide sequence of the temperature sensitive replication control region segment contained in p48K, which

25   functions in coryneform bacteria, is shown in SEQ ID NO: 7. Further, the amino acid sequences encoded by ORFs contained in these nucleotide sequences are shown in SEQ

ID NOS: 6 and 8. In the temperature sensitive
replication control region, the 1255th C is mutated to T,
the 1534th C to T, the 1866th G to A, the 2058th G to A,
the 2187th C to T and 3193rd G to A. Among these, only
5   the mutation at 1534th position is accompanied by an
amino acid mutation, and causes substitution of serine
for proline.

<5> Construction of shuttle vectors having temperature
10   sensitive mutation

Each one of the six mutations of p48K was
introduced into a shuttle vector pSFK6 (see Fig. 3).
The introduction of the mutations was performed by a
known method [Mikaelian, I., Sergeant, A., *Nucleic Acids*
15   *Res.*, *20*, 376 (1992)]. Specific procedure will be
mentioned below. In order to introduce the mutation of
1534th C to T, PCR was performed by using a combination
of the primers shown in SEQ ID NOS: 9 and 10 (primers 9
and 10), and a combination of the primers shown in SEQ
20   ID NOS: 11 and 12 (primers 11 and 12), and pAM330 as a
template. Each of the obtained amplification products
was purified by subjecting them to agarose gel
electrophoresis, and collecting them from the gel. The
collection of the DNA fragments from the gel was
25   performed by using EASYTRAP Ver.2 (Takara Shuzo Co.,
Ltd.). The purified DNAs were mixed in a molar ratio of
1:1, and used as a template for PCR performed by using

the primers shown SEQ ID NOS: 13 and 14 (primers 13 and
14). The amplification product was fully digested with
a restriction enzyme MluI, and subjected to agarose gel
electrophoresis to recover a DNA fragment of about 3.2
5   kb. Similarly, pSFK6 was also completely digested with
a restriction enzyme MluI, and subjected to agarose gel
electrophoresis to recover a DNA fragment of about 3.8
kb. The obtained DNA fragments were mixed and ligated,
and used to transform competent cells of *Escherichia*
10   *coli* JM109 (Takara Shuzo Co., Ltd.). The cells were
applied on L medium containing 25 µg/ml of kanamycin,
and cultured overnight. The appeared colonies were
picked up, and isolated single colonies to obtain
transformant strains. A plasmid was prepared from the
15   transformant strains by the alkaline method, and the
nucleotide sequence of the plasmid was determined to
confirm that 1534th C in the sequence shown in SEQ ID
NO: 5 was mutated to T. This plasmid was designated as
pSFKT2 (Fig. 3).

20

Example 2: Cloning of *argR* gene and amplification effect
thereof in coryneform bacteria

PCR was performed by using chromosome DNA of the
25   *Brevibacterium flavum* wild strain 2247 (AJ14067) as a
template and the oligonucleotides having the nucleotide
sequences shown in SEQ ID NO: 15 (sequence of the

nucleotide numbers 1717-1741 in SEQ ID NO: 17) and SEQ

ID NO: 16 (sequence complementary to the sequence of the

nucleotide numbers 2386-2362 in SEQ ID NO: 17) as

primers (Primers 15 and 16). PCR was performed for 30

5   cycles with each cycle consisting of reactions at 98°C

for 10 seconds, 58°C for 1 minute and 72°C for 3 minutes

by using Pyrobest DNA polymerase (Takara Shuzo Co.,

Ltd.). The obtained amplified fragment was inserted

into the *Sma*I site of the shuttle vector pSFK6 obtained

10   in Example 1 to obtain plasmid pWR autonomously

replicable in coryneform bacteria.

In order to investigate the amplification effect

of *argR* gene in L-arginine producing coryneform bacteria,

pWR was introduced into the AJ113455 strain (FERM BP-

15   6894), which is an L-arginine producer of *Brevibacterium*

flavum. The plasmid was introduced by the electric

pulse method (Japanese Patent Laid-open No. 2-207791).

A transformant was selected as a kanamycin resistant

strain on CM2G agar medium (containing 5 g of glucose, 5

20   g of NaCl and 15 g of agar in 1 L of pure water, pH 7.2)

containing 25 μg/ml of kanamycin to obtain AJ11345/pWR.

As a control, pSFK6 was similarly introduced into the

AJ113455 strain to obtain a transformant AJ11345/pSFK6.

Each of the aforementioned strains was plated on

25   an agar medium containing 0.5 g/dl of glucose, 1 g/dl of

polypeptone, 1 g/dl of yeast extract and 0.5 g/dl of

NaCl, and cultured at 31.5°C for 20 hours. One platunum

loop of the obtained cells were inoculated into a medium

containing 4 g/dl of glucose, 6.5 g/dl of ammonium

sulfate, 0.1 g/dl of KH_2PO_4, 0.04 g/dl of $MgSO_4$, 0.001

g/dl of $FeSO_4$, 0.001 g/dl of $MnSO_4$, 5 μg/dl of vitamin B_1,

5 5 μg/dl of biotin and soybean protein hydrolysate (45

mg/dl as N amount), and cultured in a flask at 31.5°C

for 50 hours with shaking. Accumulation amount of L-

arginine (concentration, g/dl) in each culture broth was

measured. The results are shown in Table 1. As a

10 result, the *argR*-amplified strain hardly accumulated L-

arginine. This demonstrated that the *argR* gene product

functioned as an arginine repressor.

Table 1

Strain	L-Arginine accumulation amount (g/dl)
AJ11345/pSFK6	1.3
AJ11345/pWR	0.2

15

The result of nucleotide sequencing for the

inserted fragment cloned in pWR is shown in SEQ ID NO:

17. An amino acid sequence that may be encoded by that

nucleotide sequence is shown in SEQ ID NO: 18.

20

Example 3: Construction of *argR*-disrupted strain of

coryneform bacterium and effect of deletion of arginine

repressor

<1> Construction of plasmid for *argR* disruption

25   PCR was performed by using chromosome DNA of a

wild strain of *Brevibacterium flavum*, 2247 strain
(AJ14067), as a template and the oligonucleotides having
the nucleotide sequences shown in SEQ ID NO: 19
(sequence of the nucleotide numbers 4-28 in SEQ ID NO:

5 17) and SEQ ID NO: 20 (sequence complementary to the
sequence of the nucleotide numbers 4230-4211 in SEQ ID
NO: 17) as primers (Primers 19 and 20). PCR was
performed for 30 cycles with each cycle consisting of
reactions at 98°C for 10 seconds, 58°C for 1 minute and

10 72°C for 3 minutes by using Pyrobest DNA polymerase
(Takara Shuzo Co., Ltd.). The obtained amplified
fragment was inserted into the *Sma*I site in a
multicloning site of cloning vector pHSG399.

In order to delete the whole ORF considered to

15 encode the arginine repressor from the inserted DNA
fragment, PCR was performed by using the
oligonucleotides having the nucleotide sequences shown
in SEQ ID NO: 21 (sequence of the nucleotide numbers
2372-2395 in SEQ ID NO: 17) and SEQ ID NO: 22 (sequence

20 complementary to the sequence of the nucleotide numbers
1851-1827 in SEQ ID NO: 17) as primers (Primers 21 and
22) and pHSG399 inserted with the amplified fragment as
a template. pssER was constructed by self-ligation of
the PCR product.

25 Then, a fragment obtained by digesting pssER with
restriction enzymes *Sma*I and *Sal*I and the temperature
sensitive plasmid pSFKT2 obtained in Example 1 and

digested with *Sma*I and *Sal*I were ligated to obtain plasmid pssERT for *argR* disruption whose autonomous replication ability in coryneform bacteria became temperature sensitive.

<2> Construction of arginine repressor deficient strain of coryneform bacterium by homologous recombination

The plasmid pssERT obtained as described above was introduced into the *Brevibacterium lactofermentum* AJ13029 strain (FERM BP-5189). The plasmid was introduced by the electric pulse method (Japanese Patent Laid-open No. 2-207791). Because autonomous replication ability of this plasmid is temperature sensitive in *Brevibacterium lactofermentum*, only strains in which this plasmid was incorporated into the chromosome by homologous recombination could be selected as kanamycin resistant strains at 34°C, which was a temperature that did not allow replication of the plasmid. Strains in which the plasmid for *argR* disruption was incorporated into a chromosome were selected as kanamycin resistant strains on a CM2G plate (containing 10 g/L of polypeptone, 10 g/L of yeast extract, 5 g/L of glucose, 5 g/L of NaCl and 15 g/L of agar in 1 L of water, pH 7.2) containing 25 µg/ml of kanamycin. At this stage, the normal *argR* gene derived from the chromosome and the *argR* gene derived from the plasmid in which OFR was deleted were present in tandem at the both sides of the

plasmid portion on the chromosome.

Then, the recombinant strains were allowed to cause homologous recombination again, and strains that became kanamycin sensitive at 34°C, which was a

5   temperature that did not allow the plasmid replication, were selected as strains in which one of the *argR* genes was deleted. These strains include strains in which the normal *argR* gene remained on the chromosome and strains in which the disrupted *argR* gene remained on the

10 chromosome. From these strains, a strain having only the disrupted *argR* gene was selected. An *argR* gene on the chromosome is determined to be the disrupted type by preparing chromosome of a strain that became kanamycin sensitive at 34°C, performing PCR utilizing the

15 chromosome as a template and the oligonucleotides having the nucleotide sequences shown in SEQ ID NOS: 19 and 20 as primers (Primers 19 and 20), and confirming that the PCR product was shorter by about 600 bp than that obtained by similarly performing PCR utilizing

20 chromosome derived from the parent strain as a template.

Direct sequencing of the PCR product of the *argR*-disrupted strain selected as described above was performed to confirm that the *argR* gene was disrupted as desired, and thus AJ13029ΔR stain was obtained.

25

<3> Production of L-arginine with *argR*-disrupted strain
The AJ13029ΔR strain was plated on an agar medium

containing 0.5 g/dl of glucose, 1 g/dl of polypeptone, 1 g/dl of yeast extract and 0.5 g/dl of NaCl, and cultured at 31.5°C for 20 hours. One platinum loop of the obtained cells were inoculated into a medium containing

5 3 g/dl of glucose, 6.5 g/dl of ammonium sulfate, 0.1 g/dl of KH_2PO_4, 0.04 g/dl of $MgSO_4$, 0.001 g/dl of $FeSO_4$, 0.001 g/dl of $MnSO_4$, 300 μg/dl of vitamin B_1, 200 μg/dl of biotin and soybean protein hydrolysate (165 mg/dl as N amount) and adjusted to pH 7.0 with NaOH, and cultured

10 at 31.5°C for 24 hours as seed culture.

The above seed culture broth was inoculated in an amount of 1 ml into a medium containing 4 g/dl of glucose, 6.5 g/dl of ammonium sulfate, 0.5 g/dl of KH_2PO_4, 0.04 g/dl of $MgSO_4$, 0.001 g/dl of $FeSO_4$, 0.01

15 g/dl of $MnSO_4$, 5 μg/dl of vitamin B_1, 5 μg/dl of biotin and soybean protein hydrolysate (45 mg/dl as N amount) and adjusted to pH 7.0 with KOH, and cultured in a flask at 31.5°C for 50 hours with shaking. Accumulation amount of L-arginine (concentration, mg/dl) in culture

20 broth of each strain was measured. The results are shown in Table 2. As a result, the *argR*-disrupted strain accumulated L-arginine in a markedly larger amount compared with the parent strain.

Table 2

Strain	L-Arginine accumulation amount (mg/dl)
AJ13029	20
AJ13029ΔR	120

(Explanation of Sequence Listing)

5   SEQ ID NO: 1: primer for amplification of kanamycin

resistance gene of *Streptococcus faecalis*

SEQ ID NO: 2: primer for amplification of kanamycin

resistance gene of *Streptococcus faecalis*

SEQ ID NO: 3: primer for amplification of vector portion

10   of pHSG399

SEQ ID NO: 4: primer for amplification of vector portion

of pHSG399

SEQ ID NO: 5: nucleotide sequence of replication control

region of pSFK6

15   SEQ ID NO: 6: amino acid sequence that may be encoded by

ORF in pSFK6

SEQ ID NO: 7: nucleotide sequence of replication control

region of p48K

SEQ ID NO: 8: amino acid sequence that may be encoded by

20   ORF in p48K

SEQ ID NO: 9: primer for 1st PCR for introducing

mutation of 1534th C to T into pSFK6

SEQ ID NO: 10: primer for 1st PCR for introducing

mutation of 1534th C to T into pSFK6

SEQ ID NO: 11: primer for 1st PCR for introducing mutation of 1534th C to T into pSFK6

SEQ ID NO: 12: primer for 1st PCR for introducing mutation of 1534th C to T into pSFK6

5   SEQ ID NO: 13: primer for 2nd PCR for introducing mutation of 1534th C to T into pSFK6

SEQ ID NO: 14: primer for 2nd PCR for introducing mutation of 1534th C to T into pSFK6

SEQ ID NO: 15: primer for *argR* gene amplification

10   SEQ ID NO: 16: primer for *argR* gene amplification

SEQ ID NO: 17: nucleotide sequence of DNA fragment containing *argR* gene

SEQ ID NO: 18: amino acid sequence that may be encoded by the above DNA fragment

15   SEQ ID NO: 19: primer for *argR* gene amplification

SEQ ID NO: 20: primer for *argR* gene amplification

SEQ ID NO: 21: primer for amplifying portions other than *argR* gene ORF of plasmid containing *argR* gene

SEQ ID NO: 22: primer for amplifying portions other than

20   *argR* gene ORF of plasmid containing *argR* gene